*Anti-Cancer Drugs 2002,* **13,** pp. 93–100

## Report

# Antitumor activity of protein kinase C inhibitors and cisplatin in human head and neck squamous cell carcinoma lines

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**Protein kinase C (PKC) plays a pivotal role in signal transduction involved in the control of cell proliferation, differentiation and apoptosis. Interference with such signaling pathways may result in altered tumor cell response to antineoplastic drugs. We investigated the effects of two selective PKC inhibitors as single agents and in combination with cisplatin in cell lines derived from squamous cell carcinomas of the head and neck (SCCHN). Safingol (Saf) is directed against the regulatory domain, whereas chelerythrine (Che) interacts with the catalytic domain of PKC. In six SCCHN cell lines (UM-SCC 11B, 14A, 14C and 22B, 8029NA, and a 5-fold cisplatin-resistant subline 8029DDP). PKC activities ranged between 1 and 158 IU/1 $\times 10^7$ cells, and they were inversely proportional to the amount of cellular epidermal growth factor receptor. Using the colorimetric MTT assay, PKC inhibitors Saf and Che showed comparable dose-dependent growth inhibition. The 50% inhibitory concentrations ($IC_{50}$) were between 3.8–8.6 $\mu$M for Saf and 8.5–13.6 $\mu$M for Che with no relationship to PKC activity or cisplatin sensitivity of the respective cell lines. Combinations of cisplatin ($IC_{50}$ = 0.4–5.8 $\mu$g/ml) and either PKC inhibitor (5 $\mu$M Saf, 10 $\mu$M Che) led to a significant decrease of cisplatin $IC_{50}$ values in most cell lines. However, comparison with theoretical additive dose–response curves showed additive rather than synergistic effects for both PKC inhibitors. [© 2002 Lippincott Williams & Wilkins.]**

*Key words:* Cisplatin, head and neck cancer, protein kinase C.

## Introduction

Protein kinase C (PKC) comprises a family of phospholipid-dependent serine/threonine kinases, including conventional, novel and atypical isoforms.[1,2] PKCs feature a catalytic domain with an ATP-binding site and a regulatory domain containing a $Ca^{2+}$-binding site. They are frequently involved as second messengers in the transduction of a variety of signals related to cell proliferation, differentiation, stress responsiveness and apoptosis.[1,2] Among these multiple interactions, major interest has been focused on the role of PKC in the course of signaling pathways triggered by the receptor of the epidermal growth factor (EGFR).[3–5] Furthermore, regulatory functions of PKC on the level of the EGFR itself through interference with ligand binding and/or receptor tyrosine kinase activity have been reported.[6,7] With regard to cancer, accumulating evidence strongly suggests the engagement of PKC in both development and progression of disease.[1,8,9] For squamous cell carcinomas of the head and neck (SCCHN), altered expression of certain PKC isoforms is supposed to play a role in carcinogenesis and to determine a more aggressive phenotype.[10]

Considerable progress has been made in the creation of effective chemotherapy regimens for the systemic treatment of SCCHN. Unfortunately, the increased response rates achieved have failed to translate into significant improvements in survival and, with the exception of simultaneous radio-chemotherapy, the application of antineoplastic drugs generally remains limited to the palliative setting.[11] Since the outgrowth of drug-resistant tumor cells is the major obstacle to successful chemotherapy, comprehensive efforts have been spent on the characterization and modulation of mechanisms which may help to escape from the cytotoxic encounter with antineoplastic agents.[12]

This work was supported by a grant of the German Research Society (DFG) to HB (grant Bi 362/1-2).

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The impact of cellular signaling pathways on properties typically misregulated in cancer, such as differentiation, proliferation and apoptosis, has led to the identification of novel targets to potentially overcome the phenomenon of drug resistance. High levels of the EGFR are frequently expressed in a number of malignancies, including SCCHN.[13–15] Since the EGFR is considered to participate in an autostimulatory pathway promoting cell proliferation and possibly acting anti-apoptotically, we and others have investigated the effects of anti-EGFR monoclonal antibodies and selective receptor tyrosine kinase inhibitor on the chemosensitivity of squamous cell carcinoma lines.[14,16,17] Several reports have indicated an impact of PKC on antineoplastic drug resistance showing increased tumor cell sensitivity to chemotherapy via the modulation of PKC activity.[8,18–25] In continuation of these experiments, the function of PKC as regulator and further downstream arbiter in the EGFR signaling cascade has prompted us to determine the effects of PKC modulators on the chemosensitivity of SCCHN *in vitro*.

Out of a panel of 30 cell lines established from various SCCHN, we selected six cell lines with a broad range in both PKC activity and EGFR protein expression. Furthermore, a drug resistant subpopulation, experimentally induced and described in previous experiments[26,27] was included. We tested the antitumor activity of two selective PKC inhibitors, safingol (Saf) and chelerythrine (Che), which are directed against the regulatory (Saf), respectively catalytic (Che), domain of the isoenzyme family.[28] Finally, we investigated the effects of combined treatment with either PKC inhibitor and cisplatin, one of the most potent antineoplastic agents in the systemic treatment of SCCHN, in order to detect subadditive, additive or synergistic antineoplastic activity.

## Materials and methods

### Cell lines and culture

SCCHN cell lines UM-SCC 11B, 14A, 14C and 22B were kindly provided by Dr TE Carey (University of Michigan, Ann Arbor, MI).[29] UM-SCC 14C was derived from the second recurrence of the tumor UM-SCC 14A. 8029NA represents a recloned population of cell line HLaC 79[30] and its 5-fold cisplatin-resistant subline 8029DDP has been established by chronic drug exposure.[26] All cells were grown as monolayers in plastic culture flasks (Greiner, Solingen, Germany) under standard conditions (37°C, 5% $CO_2$, fully humidified atmosphere) using modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (both Gibco, Eggenstein, Germany), 2 mM L-glutamine, 50 $\mu$g/ml streptomycin and 50 IU/ml penicillin (all ICN, Meckenheim, Germany) as described previously.[31] To transfer or passage the cell lines, almost confluent monolayers were detached with 0.05% trypsin/0.02% EDTA solution (Boehringer, Mannheim, Germany). Subsequently, cells were washed twice in medium and resuspended in 96-well flat-bottom microtiter plates (Becton Dickinson, Heidelberg, Germany) or culture flasks.

### Reagents

Cisplatin was obtained from Bristol-Myers Squibb (Munich, Germany). Two selective PKC inhibitors were used: Saf (safingol: L-threo-dihydrosphingosine, a friendly gift of Eli Lilly Company, Indianapolis, IN) is directed against the regulatory domain and Che(chelerythrine: 1,2-dimethoxy-*N*-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride; Sigma, Munich, Germany) interacts with the catalytic domain of the enzyme family members.

### PKC assay

PKC activity of tumor cells was measured with the non-radioactive Mesacup protein kinase assay (Calbiochem, Bad Soden, Germany) in three independent experiments. After subculturing, cells were grown for 6 days without reaching confluency and harvested mechanically. Employing $1–2 \times 10^7$ cells, PKC was activated with phosphatidylserine/$Ca^{2+}$ and supplementation of ATP led to phosphorylation of the pseudosubstrate precoated on 96-microwell plates. Biotinylated monoclonal antibody 2B9 binding to the phosphorylated pseudosubstrate was subsequently detected with streptavidin conjugated to peroxidase. Converted substrate was measured at 492 nm in a 96-well multireader spectrophotometer (Ear 400 ATX; SLT Labinstruments, Crailsheim, Germany). Rat brain PKC (Boehringer) served for the establishment of standard curves. PKC activity in $\mu$U was referred to $1 \times 10^7$ cells.

### EGFR assay

As described previously,[14,32] EGFR protein was determined in a sandwich-type immunoassay (Onco-

gene Science, Uniondale, NY) using mouse monoclonal capture antibody specific for the extracellular domain of the human EGFR and biotinylated goat monoclonal detector antibody. The amount of EGFR in fmol/mg total protein (Protein microassay; BioRad, Munich, Germany) was determined in three separate experiments using freshly harvested cells.

### *In vitro* growth inhibition/cytotoxicity assay

Antitumor effects were determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) test. This colorimetric assay is based on the reduction of a non-toxic water-soluble yellow tetrazolium salt to a purple-colored water-insoluble formazan precipitate by the reductive capacity of cytoplasmatic and mitochondrial dehydrogenases present only in living metabolically active cells. Exponentially growing tumor cells were harvested from culture flasks, plated in 96-well microtiter plates (6000 cells/200 $\mu$l culture medium/well) on day 0 and incubated for 72 h. On day 3, fresh culture medium supplemented with different concentrations of cisplatin, PKC inhibitor or combinations of cisplatin and PKC inhibitor was added, and controls received medium alone. On day 6, 5 $\mu$g MTT solution was added to each well and left to react for 4 h. After the complete dissolving of formazan precipitates in 150 $\mu$l dimethylsulfoxide (Sigma), absorbance was measured at 570 nm in a microplate reader (Ear 400 ATX). Wells with all components of the mixtures except cells served as blanks. In previous investigations, this experimental procedure has been identified to produce a good linear correlation between absorbance and the number of viable tumor cells.[14,32] Therefore, tumor cell survival as a percentage of the control (fractional absorbance) was calculated according to the formula: [(absorbance test – absorbance blank)/(absorbance control–absorbance blank)] $\times$ 100.

All MTT assays were performed as 6-fold determinations and the coefficient of variation ($V_c$=standard deviation $\times$ 1/mean value $\times$ 100) of repeats never exceeded 10%. The 50% inhibitory drug concentrations ($IC_{50}$) for cisplatin and/or PKC inhibitors were determined in three independent experiments employing a non-linear estimation procedure (SAS-PC 6.08; SAS Institute, Carey, MI) resulting in sigmoid dose–response curves.[31,33] Mean $IC_{50}$ concentrations of Saf (5 $\mu$M) and Che (10 $\mu$M), and five escalating concentrations of cisplatin were employed for combined drug exposure To distinguish additive, subadditive and synergistic effects, we established theoretical additive dose–response curves according to the logit transformation method of Pöch and Holzmann.[34]

In order to obtain information on the mode of antitumor action of the PKC inhibitors, we looked for apoptosis in two selected SCCHN cell lines: 8029NA (high PKC activity) and UM-SCC 14 C (low PKC activity). Similar as described previously,[35] apoptotic cells were detected with both ELISA (Cell Death Detection plus; Boehringer, Mannheim, Germany) and light microscopy (H & E staining) at 4, 24, 48 and 72 h after the addition of Saf, Che and/or cisplatin. Controls remained untreated. The ELISA is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, which allows specific detection of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. Results obtained in the ELISA are shown as intensification factor (IR), defined as: IR=extinction in experimental group/extinction in control group.

In addition, using light microscopy, 1000 cells of both experimental and control groups were evaluated by KL and CDG in a blinded fashion for typical morphological criteria of apoptosis.

## Results

### PKC activity and EGFR expression

For the six SCCHN lines included in this study, PKC activity ranged between 1 and 158 $\mu$U/1 $\times$ $10^7$ cells,

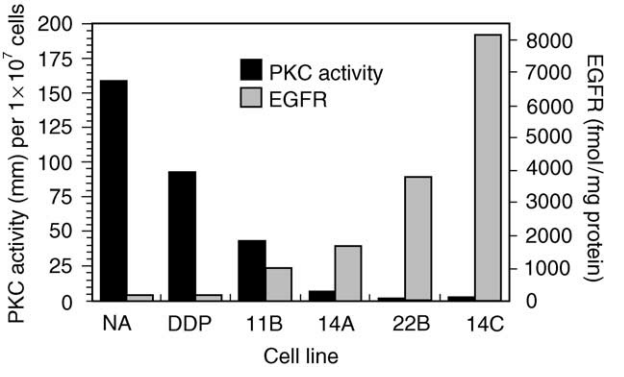


**Figure 1.** Inverse relationship between PKC activity and EGFR expression in six SCCHN lines. Mean PKC activity ranged between 1 and 158 IU/1 $\times$ $10^7$ cells (left *y*-axis) in cell lines UM-SCC 11B, 14A, 14C, 22B, 8029NA and 8029DDP, and was inversely proportional to the epidermal growth factor receptor status (170–8100 fmol/mg protein, right *y*-axis). Results are the mean of three independent determinations and standard deviations were below 15%.

and the amount of EGFR protein ranged between 20 and 8100 fmol/mg protein (Figure 1). The highly EGFR expressing tumors UM-SCC 14C, 22B and 14A (8100, 3600 and 1800 fmol/mg protein) exhibited low PKC activities (2, 1 and 7 $\mu$U/1 $\times 10^7$ cells), whereas low amounts of EGFR in 8029NA, 8029DDP and UM-SCC 11B (170, 170 and 1000 fmol/mg protein) were associated with high enzyme activities (158, 92 and 43 $\mu$U/1 $\times 10^7$ cells). This difference was found to be statistically significant (Spearman rank correlation with $r = -0.9276$ and $p = 0.0167$).

### Growth inhibition/cytotoxicity

Both PKC inhibitors showed a strong dose-dependent growth inhibition with $IC_{50}$ values between 8.5. and 13.6 $\mu$M (mean 10.3 $\mu$M) for Che and 3.8 and 8.6 $\mu$M (mean 5.8 $\mu$M) for Saf (Table 1). $IC_{50}$ values of cisplatin confirmed the data obtained in earlier experiments: UM-SCC 11B 0.8 $\mu$g/ml; UM-SCC 14A 0.4 $\mu$g/ml; UM-SCC 14C 0.8 $\mu$g/ml; UM-SCC 22B 1.1 $\mu$g/ml; 8029NA 1.2 $\mu$g/ml; 8029DDP 5.8 $\mu$g/ml. No correlation between PKC activity and growth inhibition through either PKC inhibitor or cisplatin was evident.

Using the mean $IC_{50}$ concentrations for Saf (5 $\mu$M) and Che (10 $\mu$M) in combination with increasing doses of cisplatin, comparison with the theoretical additive dose–response curves demonstrated additive rather than synergistic effects of combined treatment in five of the six cell lines tested (UM-SCC 11B, 14A, 14C, 22B and 8029NA). Both PKC inhibitors exhibited only subadditive activity in the cisplatin resistant subline 8029DDP (Figure 2).

Saf and Che induced apoptosis in all SCCHN lines tested. For both PKC inhibitors, the number of apoptotic cells was generally enhanced by prolonged exposure, increased concentration and combined treatment with cisplatin. Cell line UM-SCC 14C (low PKC activity) developed the maximal number of apoptotic cells after exposure to Saf for 24 h, to Che for 24–48 h and to cisplatin for 48 h. The combination of cisplatin and Saf induced a maximum of apoptotic cells after 48–72 h, whereas for cisplatin and Che the peak was detected after 24–48 h (Figure 3). For cell line 8029NA (high PKC activity) maximum of apoptosis was at 24–48 h for Saf and at 48 h for Che as well as for cisplatin. For both combinations of cisplatin with either Saf or Che, the maximum of apoptotic cells was detected at 48 h (Figure 4). However, for Che but not for Saf combined treatment with cisplatin was always less effective in inducing apoptosis compared to Che alone (Figure 4).

**Table 1**. PKC activities and 50% inhibitory concentrations ($IC_{50}$) for Che, Saf and Cis

| Cell line | PKC ($\mu$U) per $1 \times 10^7$ cells | $IC_{50}$ Che ($\mu$M) | $IC_{50}$ Saf ($\mu$M) | $IC_{50}$ Cis ($\mu$g/ml) |
|---|---|---|---|---|
| 8029NA | 158 | 10.8 | 7.0 | 1.2 |
| 8029DDP | 92 | 13.6 | 5.7 | 5.8 |
| UM-SCC 11B | 43 | 10.3 | 4.1 | 0.8 |
| UM-SCC 14A | 7 | 9.3 | 5.4 | 0.4 |
| UM-SCC 22B | 1 | 9.3 | 3.8 | 1.1 |
| UM-SCC 14C | 2 | 8.5 | 8.6 | 0.8 |

## Discussion

The PKC family represents a group of pivotal, enzymatically active constituents in numerous signaling pathways involved in the regulation of cellular homeostasis, including differentiation, proliferation and apoptosis. In the present panel of six SCCHN cell lines we found considerable differences in PKC activities ranging from 1 to 158 $\mu$U/$10^7$ cells. Interestingly, these isoenzyme activities showed an impressive inverse relationship to the levels of EGFR expression: low PKC activity was associated with high receptor protein expression and vice versa. Since PKC has appeared to be an important mediator of EGFR endocytosis and degradation,[36,37] it may be speculated that marked EGFR expression in SCCHN partly results from inadequate, endogenously controlled receptor down-regulation, internalization or cleavage.[38,39]

We found different PKC activities in the recloned cisplatin sensitive cell line 8029NA (158 $\mu$U/$1 \times 10^7$ cells) and the 5-fold cisplatin-resistant subpopulation 8029DDP (92 $\mu$U/1 $10^7$ cells). This decrease, however, is in contrast to a report by Basu and Weixel[21] describing 2-fold increased PKC activity in cisplatin resistant ovarian carcinoma cells in comparison to the corresponding drug sensitive progenitor cells. Furthermore, in the panel of HNSCC lines investigated in the current study, we have no indication for a direct relationship between PKC activity, on the one hand, and cisplatin resistance, on the other. Only recently, Hofmann[8] thoroughly reviewed the literature on the impact of PKC activity on the chemosensitivity of tumor cells, and he emphasized the great number of conflicting results obtained in various *in vitro* and *in vivo* models. In this context, the involvement and, finally,

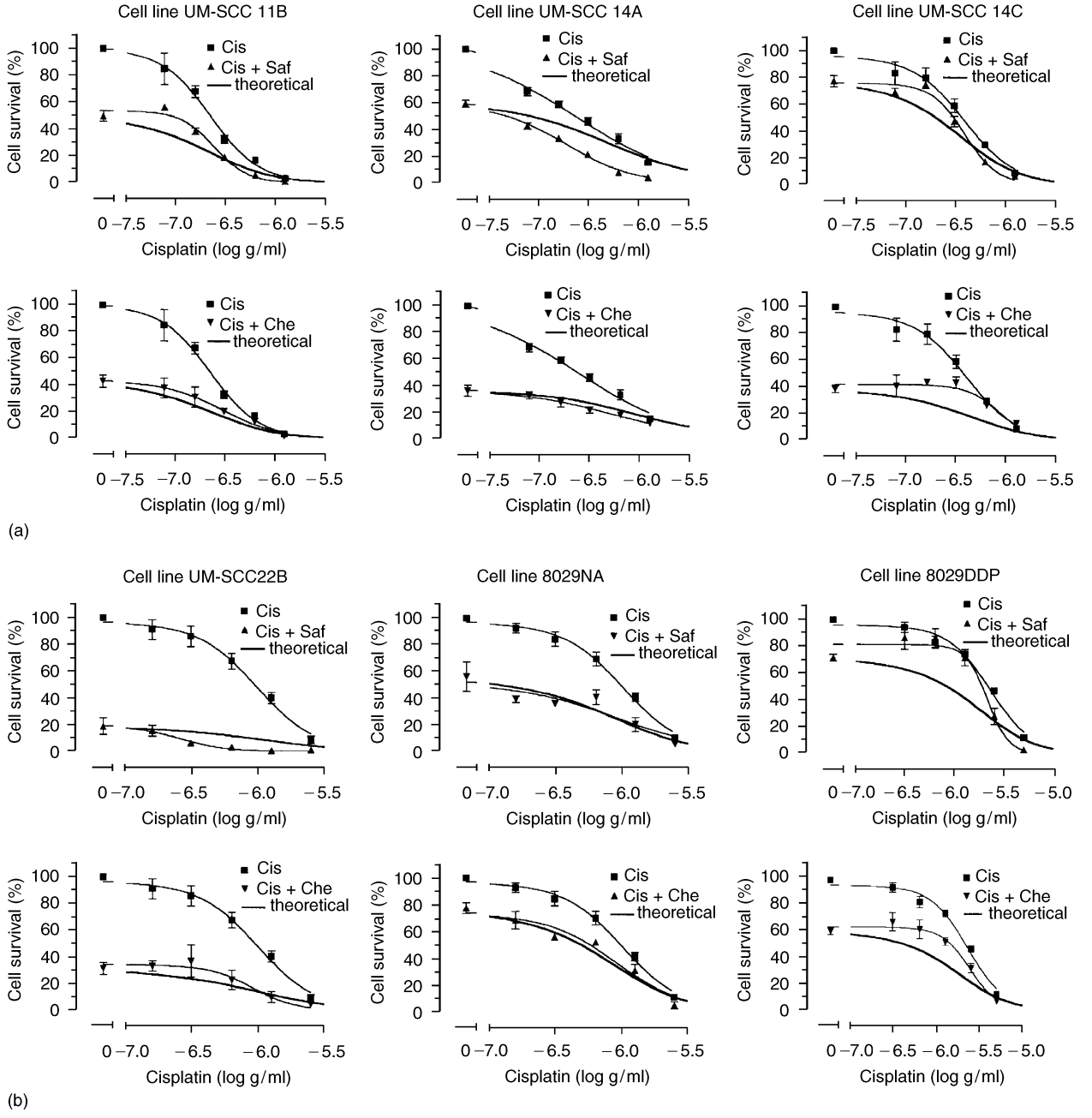


**Figure 2.** Analysis of additive, synergistic and subadditive effects for combinations of PKC inhibitors with cisplatin. Using mean $IC_{50}$ concentrations for Saf (5 $\mu$M) or Che (10 $\mu$M) in combination with five increasing doses of cisplatin, analytical comparison with the corresponding theoretical additive dose–response curves was performed. The *x*-axis shows log transformed data and the *y*-axis survival of cells in percent. Vertical bars within the curves represent standard deviations of three independent experiments performed with six repeats each.

the modulation of distinct PKC isoforms may be of major importance.

Since many inhibitory compounds interfere with protein kinases indiscriminately, we used two specific PKC inhibitors interacting with the regulatory (Saf) and catalytic (Che) domain, respectively.[9,28] In all our cell lines, Saf and Che showed a strong dose-dependent growth inhibition accompanied by a marked increase in the number of apoptotic tumor cells. Similar antitumor activity of PKC inhibitors has been described in a variety of cancers, including SCCHN.[40–42] However, the decision between proliferation and growth inhibition/apoptosis is likely to depend on factors

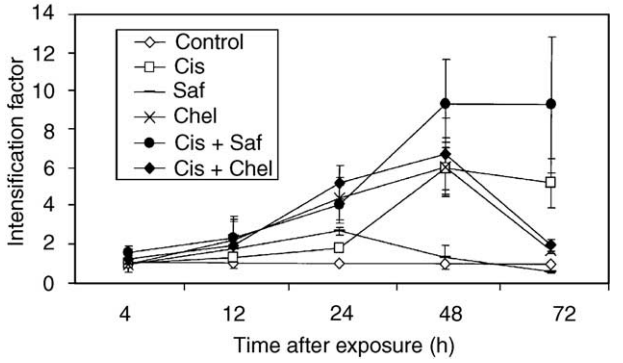


**Figure 3.** Evaluation of apoptosis by ELISA. Representative data of cell line UM-SCC 14C (high EGFR expression, low PKC activity) are shown, cells were untreated (control) or treated with 0.625 $\mu$g/ml cisplatin (Cis), 5 $\mu$M Saf, 10 $\mu$M Che and their combinations. The intensification factor was defined as extinction of experimental group/extinction of control group ($n = 2$, vertical bars represent standard deviations).

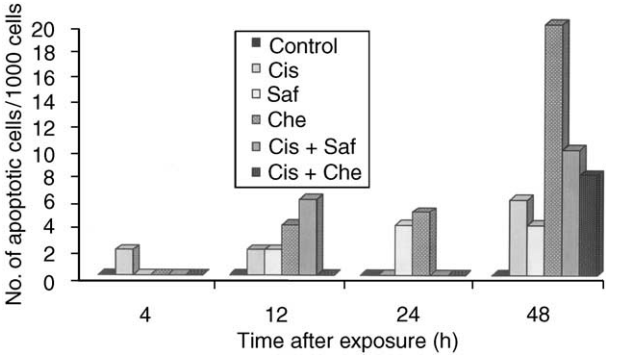


**Figure 4.** Evaluation of apoptosis by morphological criteria. Representative data of cell line 8029NA (low EGFR expression, high PKC activity). Cells were untreated (control) or treated with 0.625 $\mu$g/ml Cisplatin (Cis), 5 $\mu$M Saf, 10 $\mu$M Che and their combinations.

such as cell type, cell cycle, cellular microenvironment, specificity of the inhibitory agent and duration of treatment.[8]

In the current study we were interested if it would be possible to modulate the sensitivity of established SCCHN cell lines to cisplatin through the selective inhibition of PKC activity with either Saf or Che. Both PKC inhibitors were found to increase the activity of cisplatin. However, this effect turned out to be only additive in five and subadditive in the experimentally induced cisplatin-resistant subpopulation 8029DDP. Recent reports have shown that in gastric cancer lines Saf is capable to potentiate the antineoplastic activity of both cisplatin and mitomycin C, also resulting in increased apoptosis.[43] These results provided the basis for clinical trials with single-dose application of 120 mg/m$^2$ Saf showing no major toxicity and a combination of Saf plus doxorubicin.[44] Saf serum levels achieved in these studies correspond well to the concentration used in our *in vitro* experiments. On the other hand, the idea of chemopotentiation with PKC inhibitors is challenged by reports on the PKC activator TPA (phorbol ester) which was found to enhance the antineoplastic activity of cisplatin as well.[20,24] This may reflect cell-type specific differences or the pleiotropic and multifunctional effects of TPA, e.g. the inhibition or depletion of PKC in some cell types.[8,23,25,45] In addition, the considerable heterogeneity of PKC family members and their complex regulation may contribute to these discordant results.[8,22]

## Conclusion

Our present study has shown increased cisplatin sensitivity in SCCHN lines through the addition of PKC inhibitors. This effect, however, is limited to additive activity only. Inhibition or activation of other signal transduction pathways such as transforming growth factor-$\alpha$, tumor necrosis factor (TNF)-$\alpha$, TNF-related apoptosis-inducing ligand and insulin-like growth factor, have been shown to also modulate cisplatin sensitivity.[15,46,47] Thus the concept is evolving that signal transduction pathways are involved in the regulation of cellular responses to drug-induced stress/damage being critical for cell survival.[48] The classical paradigm that drug resistance is primarily determined by inadequate drug–target interactions undergoes significant modifications as subsequent events gain decisive importance for the fate of the cells. So far, however, protocols including antineoplastic drugs and modulators of relevant signal transduction pathways with supradditive/synergistic activities remain to be established.

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*(Received 2 October 2001; accepted 16 October 2001)*